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The Drosophila *smoothened* Gene Encodes a Seven-Pass Membrane Protein, a Putative Receptor for the Hedgehog Signal

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Summary

***smoothened* (*smo*) is a segment polarity gene required for correct patterning of every segment in Drosophila. The earliest defect in *smo* mutant embryos is loss of expression of the Hedgehog-responsive gene *wingless* between 1 and 2 hr after gastrulation. Since *smo* mutant embryos cannot respond to exogenous Hedgehog (Hh) but can respond to exogenous Wingless, the Smo product functions in Hh signaling. Smo acts downstream of or in parallel to Patched, an antagonist of the Hh signal. The *smo* gene encodes an integral membrane protein with characteristics of G protein–coupled receptors and shows homology to the Drosophila Frizzled protein. Based on its predicted physical characteristics and on its position in the Hh signaling pathway, we suggest that *smo* encodes a receptor for the Hh signal.**

Introduction

The development of a multicellular organism depends on mechanisms that initially specify and subsequently maintain positional information. In Drosophila embryos, a cascade of transcription factors progressively defines position along the antero-posterior axis with increasing precision before cellular blastoderm (Nüsslein-Volhard and Wieschaus, 1980; reviewed by Small and Levine, 1991; St Johnston and Nüsslein-Volhard, 1992). At cellular blastoderm and later stages of development, information is maintained across cell borders by signal transduction pathways, the components of which are encoded by segment polarity genes (reviewed by Peifer and Bejsovec, 1992). Two such pathways are initiated by the extracellular signals Wingless (Wg) and Hedgehog (Hh). These secreted proteins (van den Heuvel et al., 1989; González et al., 1991; Lee et al., 1992; Taylor et al., 1993; Tabata and Kornberg, 1994) are synthesized in adjacent stripes of cells to delineate and maintain the parasegmental boundaries, which initially define the metameric pattern in the larva and the adult (Martinez-Arias and Lawrence, 1985). Expression of Wg and Hh are tightly linked during gastrulation and germ band extension. During these stages, Wg maintains, through a series of partially known steps, expression of the homeodomain transcription factor Engrailed (En; Siegfried et al., 1992, 1994), which represses the *cubitus interruptus* (*ci*) gene (Eaton and Kornberg, 1990) encoding a zinc-finger transcription factor (Orenic et al., 1990). Since the Ci protein represses *hh*, repression of *ci* by En activates *hh* transcription (Domínguez et al., 1996). The secreted Hh protein in turn triggers a signal transduction cascade that activates *wg* (DiNardo et al., 1988; Hidalgo and Ingham, 1990; Ingham, 1993; Ingham and Hidalgo, 1993) and at least another segment polarity gene, *patched* (*ptc*; Hidalgo and Ingham, 1990; Tabata and Kornberg, 1994). Transcription of *wg* is further stimulated in an autocrine loop by its own protein product (Hooper, 1994; Yoffe et al., 1995). The short range of both signals dictates that *wg* and *hh* expression patterns are restricted to adjacent narrow stripes of cells (Vincent and Lawrence, 1994; Porter et al., 1995). Anything that interferes with either signal quickly leads to loss of the other signal and to catastrophic failure of segmentation. By the extended germ band stage, expression of *wg* and *hh* is no longer linked since *en* becomes autoregulatory (Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991), and *wg* is maintained by an autocrine loop (Li and Noll, 1993). As development proceeds, Hh and Wg signals act as morphogens to specify many aspects of the larval cuticle pattern (Bejsovec and Wieschaus, 1993; Heemskerk and DiNardo, 1994).

Although some of the functions of these signals in morphogenesis are known, the receptors required to transduce them have remained elusive. Candidates for the receptors might be among the segment polarity genes that have not yet been isolated and whose phenotypes resemble that of *hh* or *wg*. One of the segment polarity genes that fulfills this criterion is *smoothened* (*smo*). Another that has been postulated to encode the Hh receptor is *ptc* (Ingham et al., 1991). The activity of the Ptc product, which is a multiple membrane-spanning cell-surface protein (Hooper and Scott, 1989; Nakano et al., 1989), represses the *wg* gene and is antagonized by the Hh signal (Ingham et al., 1991). However, it cannot be the only Hh receptor, since Hh has effects in *ptc* null embryos (Bejsovec and Wieschaus, 1993). Other segment polarity gene products implicated in the Hh signal transduction pathway but clearly not functioning as Hh receptor are the zinc-finger protein Ci and two serine/threonine protein kinases, Fused (Fu) and cyclic AMP–dependent protein kinase A (PKA; reviewed by Perrimon, 1995).

This study describes a genetic and molecular characterization of the *smo* gene. We show that *smo* is necessary for Hh signaling and that it acts downstream of *hh* and *ptc*. It encodes a protein with many structural features of the serpentine family of heterotrimeric G protein–coupled receptors and is homologous to the Drosophila *frizzled* gene. Based on genetic and molecular analyses, we propose that Smo is the receptor of the Hh signal.

Results

The *smoothened* Segment Polarity Phenotype

The *smo* gene was identified as a segment polarity gene and initially named *smooth* (Nüsslein-Volhard et al.,

1984). Since this name already described another locus, the new segment polarity gene was renamed *smoothened* (Lindsley and Zimm, 1992). Nüsslein-Volhard et al. (1984) recovered three recessive zygotic lethal alleles of *smo*, which are all cold-sensitive. At 25°C, the morphological defects are mild, while at 18°C, *smo* embryos exhibit a classic segment polarity cuticle phenotype (Nüsslein-Volhard et al., 1984). There is variability in the severity of the cuticle phenotype. Some individuals have lost most segmental modulation apparent in the wild type and resemble *hh* or *wg* null embryos (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984). Others retain considerable segmental modulation of denticle type and polarity (data not shown).

The variability and cold sensitivity suggest that these embryos retain residual *smo* function, possibly because the alleles are not null or because of a maternal effect. It is difficult to assess the *smo* null phenotype, since the available deficiencies that uncover *smo* do not survive beyond early embryonic stages. However, smo^{1}/Df and smo^{3}/Df exhibit a range and frequency of phenotypes similar to homozygotes and transheterozygotes of all three *smo* alleles, suggesting that these behave as near null alleles at 18°C. Although we have not tested whether a maternal contribution of smo^{+} to the developing oocyte accounts for the phenotypic variability of homozygous or hemizygous *smo* embryos, the considerable levels of *smo* mRNA in 0–2 hr embryos revealed by Northern blots (data not shown) renders this explanation plausible. The remaining analysis presented here reports phenotypes seen in the majority of *smo* mutant embryos raised at 18°C.

A classic segment polarity cuticular phenotype, such as that of *smo* mutant embryos, predicts loss of Wg and Hh signaling at the parasegment border in the ectoderm before stage 11 (reviewed by Perrimon, 1994). After their initial activation by pair–rule gene products in neighboring stripes of epidermal cells, Wg and Hh expression become interdependent. In the absence of Wg signaling, e.g., in wg^{-} embryos, loss of En protein slightly precedes that of Wg during stage 9 and early stage 10 (Figures 1A and 1B; DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991; Hidalgo, 1991; Peifer et al., 1991; Ingham and Hidalgo, 1993), because Wg signaling directly stimulates the expression of both genes (Bejsovec and Martinez Arias, 1991; Hooper, 1994; Yoffe et al., 1995). In the absence of Hh signaling, e.g., in hh^{-} embryos, Wg protein is lost during stage 9 (Hidalgo and Ingham, 1990; Hidalgo, 1991; Ingham and Hidalgo, 1993), followed by loss of En ventrally and dorsally during late stage 9 and early stage 10 (DiNardo et al., 1988). Thus, in hh^{-} embryos, Wg protein has nearly completely decayed by late stage 9, when En protein only begins to disappear (Figure 1C). In *smo* mutant embryos, Wg and En expression are initiated normally at the cellular blastoderm stage (data not shown). Wg protein subsequently decays during stage 9 (van den Heuvel et al., 1993), clearly before En protein begins to decay (Figure 1D). Therefore, the temporal loss of En protein in *smo* embryos resembles that in *hh* embryos and is different from that in *wg* embryos, consistent with a role for Smo in Hh rather than paracrine Wg signaling.

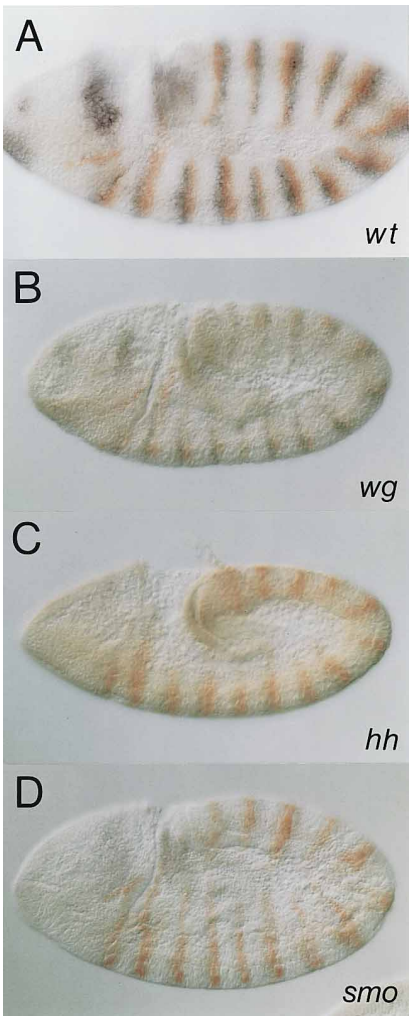


Figure 1. The *smoothened* Phenotype Resembles the *hedgehog* Rather Than the *wingless* Phenotype

Lateral epidermal expression patterns of the Wg protein (black) and the En protein (orange) are shown for wild-type (A), wg^{IL114}(B), hh^{IJ35}(C), and smo^{2} (D) embryos at late stage 9 (B–D) or early stage 10 (A) (Campos-Ortega and Hartenstein, 1985). En decays earlier in *wg* embryos than in *hh* and *smo* embryos.

smoothened Acts in Hh or Autocrine Wg Signal Transduction

To assess the requirement for smo^{+} function in Wg versus Hh signaling, we have expressed each of these signals ectopically, under the control of heterologous promoters in *smo* mutant embryos. When Wg is expressed ectopically under the control of the pair–rule *hairy* promoter in alternate parasegments of *hGAL4 UASwg* embryos, it drives ectopic expression of En in odd-numbered stripes that, as compared to wild-type En stripes (Figure 2A), are expanded posteriorly (Figure 2B; Yoffe et al., 1995), even in the absence of a functional Smo protein (Figure 2C). Thus, smo^{+} is not essential for this response to paracrine Wg signaling. Even-numbered En stripes are unaffected by the ectopic Wg signal, as expected (Figures 2B and 2C). In *smo* embryos, all En bands eventually decay (Figures 2C and 2D).

When Hh is ubiquitously expressed under the control

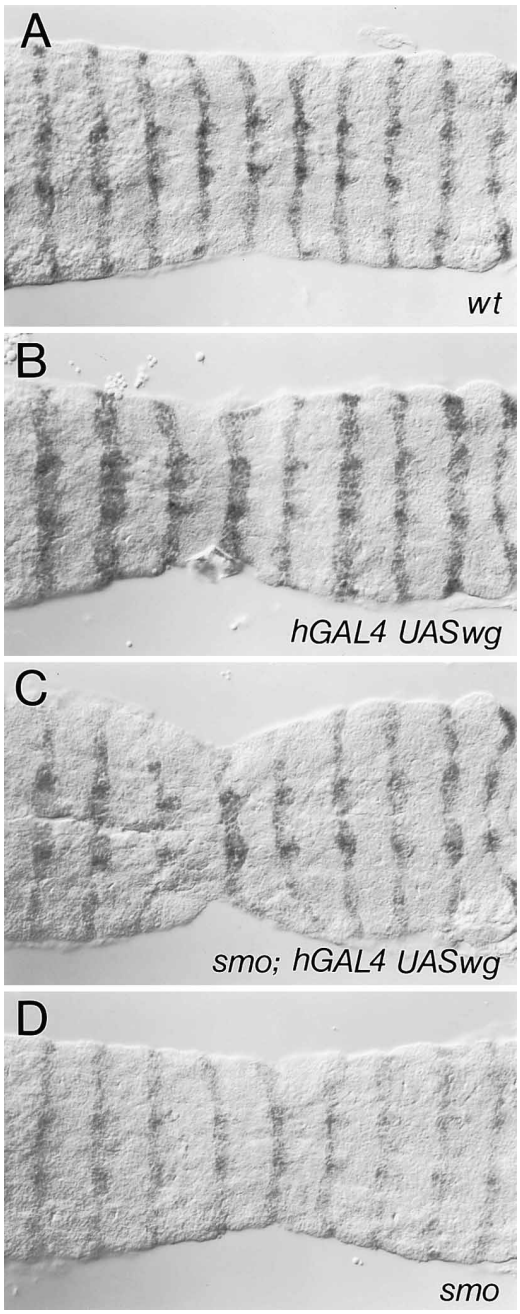
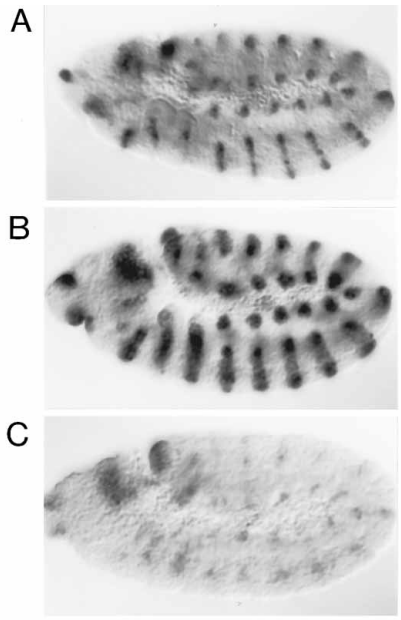


Figure 2. Ectopic Expression of Wg Activates Ectopic En Expression in Both Wild-Type and *smo* Embryos

En protein expression is shown in wild-type (A), *hGAL4 UASwg* (B), *smo*^{2}/*smo*^{3}; *hGAL4 UASwg* (C), and *smo*^{2}/*smo*^{3} embryos (D). Compared to wild-type embryos (A), odd-numbered En stripes are expanded posteriorly in *hGAL4 UASwg* embryos (B–C) because of ectopic Wg expression in odd-numbered parasegments (Yoffe et al., 1995). In *smo*^{2}/*smo*^{3}; *hGAL4 UASwg* embryos (C), even parasegments show the weak En expression characteristic of *smo* mutants (D). Thus, Wg protein can induce a response in *smo* mutant embryos. Stage 11 embryos were cut along the amnioserosa, unfolded, and flattened to show En expression in the epidermis of parasegments 5–14.



D

phenotypes of progeny	expected % of embryos if Smo is in Hh pathway	expected % of embryos if Smo is in Wg pathway	observed embryos N	observed embryos %
wild type	37.5	37.5	92	41 ± 5
Hshh	37.5	50	82	37 ± 5
smo^{-}	25	12.5	49	22 ± 5

Figure 3. Ubiquitous Hh Expression Cannot Activate Wg Expression in *smo* Embryos

Wg expression patterns of wild-type (A), heat-shocked *Hshh* (B), and *smo*^{2}/*smo*^{3} whole-mount embryos (C) are shown at stage 11. The expected ratios of the three different phenotypes (wild-type narrow Wg stripes, *Hshh*–induced broad Wg stripes, and decay of Wg protein characteristic of *smo* embryos) among the progeny of a cross between *smo*^{2}/*CyO* and *smo*^{3}/+; *Hshh*/+ flies are listed in (D) for *smo* being in either the Hh or Wg signaling pathway. Statistical analysis of the number (N) and relative ratios (including standard deviations) of observed Wg expression phenotypes indicate that Smo is in the Hh rather than in the Wg signaling pathway.

of a heat-inducible promoter (*Hs-hh*), an immediate response is the ectopic expression of *wg* in anteriorly expanded stripes (Figures 3A and 3B; Ingham, 1993; Tabata and Kornberg, 1994). In *smo* mutant embryos, ubiquitously expressed Hh does not induce ectopic expression of *wg*. Instead, Wg stripes decay and *wg* expression is indistinguishable from that of *smo* mutants in the absence of ectopic Hh expression (Figure 3C). This conclusion is derived from a statistical analysis of mixed populations of wild-type, *Hs-hh, smo*, and *smo Hs-hh* embryos (Figure 3D). Since ectopic Hh has little or no effect in *smo* mutants, *smo*$^{+}$ is required for Hh signal transduction unless the observed anterior expansion of Wg stripes also depends on an autocrine Wg signal. In this case, the lack of a role of *smo* in paracrine Wg signaling, demonstrated by the experiments shown in Figures 1 and 2, does not strictly exclude the formal

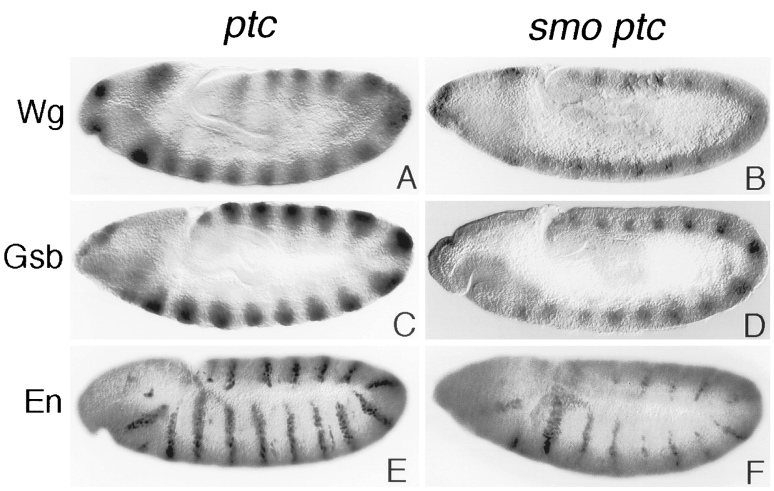


Figure 4. *smoothened* Is Epistatic to *patched*

Mid-sagittal *wg* RNA (A, B), mid-sagittal Gsb protein (C, D), and lateral surface En protein expression patterns are shown in ptc^{IN} (A, C, E) and smo^{1} *ptcDf* (B, D, F) mutant embryos at stage 9/10. In *ptc* mutant embryos, *wg* RNA and Gsb protein expression expand anteriorly to fill the posterior half of each parasegment. The ectopic Wg then induces discontinuous ectopic stripes of En expression in the middle of the parasegment. In *smo ptc* embryos, ectodermal *wg*, Gsb, and En expression fade during stages 9 and 10, as in *smo* embryos.

possibility that smo^{+} is essential in autocrine Wg signal transduction.

smoothened Acts Downstream of or in Parallel to *patched*

The *patched* (*ptc*) gene encodes an integral membrane protein of the cell surface (Hooper and Scott, 1989; Nakano et al., 1989), which has been proposed to encode the Hh receptor (Ingham et al., 1991). To investigate whether *smo* acts upstream, downstream, or in parallel to *ptc*, we have analyzed the phenotype of *smo ptc* double mutant embryos. In the absence of *ptc* activity, both Wg and Gsb expression expand anteriorly to fill half the parasegment (Figures 4A and 4C; Martinez Arias et al., 1988; Hidalgo, 1991). The expanded Wg expression may induce ectopic En expression in the middle of the parasegments anterior to its own expression (Figure 4E; DiNardo et al., 1988). In *smo ptc* double mutant embryos, Wg and Gsb expression are lost in the ectoderm (Figures 4B and 4D), and En expression is partially lost (Figure 4F), just as in *smo* mutant embryos (Figures 1D and 2D). Thus, *smo* loss of function is epistatic to *ptc* loss of function, which suggests that smo^{+} acts downstream of or in parallel to ptc^{+} in Hh signal transduction.

Cloning and Identification of the *smoothened* Gene

By meiotic recombination, *smo* maps distal to *aristaless* (*al*; 2–0.4) and *dumpy* (*dp*; 2–13.0). A single *smo al* recombinant was obtained from 44 recombinant chromosomes recovered from smo^{+} *al dp*/*smo* al^{+} dp^{+} mothers. That recombinant also carried *dp*, placing *smo* outside of and distal to the *al–dp* interval, since recombination between *smo* and *dp* occurred about 20 times more frequently. Consistent with this location of *smo*, duplication mapping places it distal to the breakpoint of *T(Y;2)L124* that is located between *expanded* (*ex*) and *u-shaped* (*ush*) in the deficiency *Df(2L)al* (P. Heitzler, personal communication). Finally, deficiency mapping narrows the region including *smo* to the interval between the breakpoint of the terminal deficiency *Df(2L)PMF* at 21B7–8 and the distal breakpoint of *Df(2L)al* at 21C1. We shall refer to this genetically defined interval as the *smo* region.

The *smo* gene was cloned by extending a chromosomal walk, comprising the deficiency *Df(2L)al* at 21C (Schneitz et al., 1993), toward the left telomere to include the breakpoint of the deficiency *Df(2L)PMF* and thus the *smo* region (Figure 5). The breakpoint of *Df(2L)PMF* was mapped to a region between −294 and −295 on the chromosomal walk, while the distal breakpoint of *Df(2L)al* is located between −257 and −260 (Schneitz et al., 1993). Thus, the genetically defined *smo* region, which must contain all sequences essential for smo^{+} function, lies between coordinates −257 and −295 (Figure 5).

To locate transcripts within this region that appear at the time when smo^{+} function is required, Northern blots of poly(A)$^{+}$ RNA from 4–8 hr old embryos were screened by hybridization with probes from across the *smo* region. At least six different transcripts were identified in 4–8 hr embryonic mRNAs within this 38 kb interval. To pinpoint the *smo* transcript, five overlapping genomic DNA fragments were introduced into the germline by P element–mediated transformation. When crossed into a smo^{-} background, only one of these, a 12.1 kb *AatII* fragment (Figure 5), complemented *smo*. More detailed mapping of isolated cDNAs corresponding to the transcripts of this genomic fragment revealed that it contained three independent transcription units. While a 9.1 kb *Aat*II–*Apa*I fragment harboring the two distal transcription units failed to complement *smo*, a 6.2 kb *Hin*dIII–*Aat*II fragment, which encodes a 4.2 kb mRNA, the 5′ end of a flanking distal transcript, and the 3′ end of a flanking proximal transcript, was found to contain all smo^{+} rescuing activity (Figure 5). One copy of this fragment fully rescued all aspects of the smo^{-} phenotype and hence includes all essential sequences for expression and function of the *smo* gene. The encoded 4.2 kb mRNA is the *smo* transcript.

Genomic and cDNA sequencing of the *smo* transcription unit reveals six exons spread over 4584 bp (Figure 5). They generate an mRNA whose length is 4005 nt without its poly(A) tail. The transcription start site was identified by two independent clones using the 5′ rapid

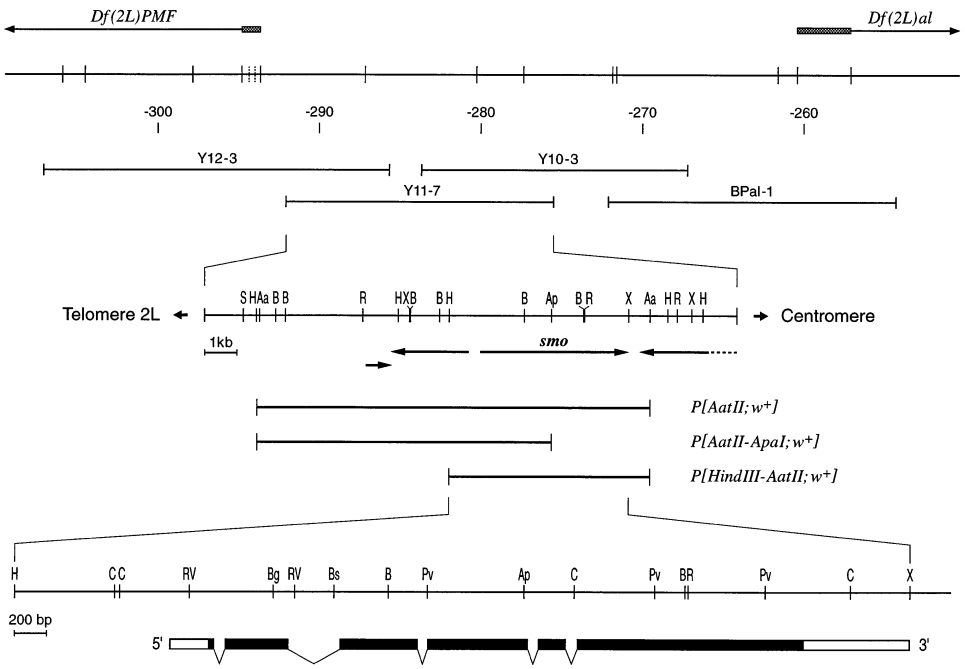


Figure 5. Cloning and Identification of the *smo* Gene

(Top) The proximal breakpoint of *Df(2L)PMF* and the distal breakpoint of *Df(2L)al* are indicated with respect to a genomic *Eco*RI map (scale in kb; broken lines are *Eco*RI sites of neighboring fragments whose order has not been determined), derived from a chromosomal walk of the region between 21B7–8 and 21C1 (Y12–3, Y11–7, Y10–3, and BPal-1 are inserts isolated from a genomic library prepared in EMBL4), a distal extension (H.-P. Lerch and M. N., unpublished data) of a walk covering the *Df(2L)al* (Schneitz et al., 1993). Stippled boxes delimit the *Eco*RI fragments that include the deficiency breakpoints.

(Middle) An enlarged map of clone Y11–7 is shown, below which the orientation and extent of the *smo* transcript and three unknown transcripts are indicated by arrows (dashed line is uncertainty of 5′ end of the most proximal transcript). Underneath, the genomic inserts of three P-element constructs that rescue (*P[AatII;w*$^+$*]* and *P[HindIII-AatII;w*$^+$*]*) or fail to rescue (*P[AatII-ApaI;w*$^+$*]*) *smo* mutants are shown.

(Bottom) A restriction map of an enlarged genomic fragment included in the P-element rescue construct *P[HindIII–AatII, w*$^+$*]* is shown with the intron/exon structure and the open reading frame (in black) of the *smo* gene. (Aa) *Aat*II; (Ap) *Apa*I; (B) *Bam*HI; (Bg) *Bgl*II; (Bs) *Bsp*EI; (C) *Cla*I; (H) *Hin*dIII; (Pv) *Pvu*II; (R) *Eco*RI; (RV) *Eco*RV; (S) *Sal*I; (X) *Xho*I.

amplification of cDNA ends (RACE) technique. A possible TATA box lies 18 bp upstream of the transcriptional start site, and the canonical polyadenylation signal AATAAA is found 17 nt upstream of the poly(A) tract.

Northern blots using staged mRNAs and in situ hybridizations to embryos and imaginal discs show that the *smo* gene is expressed at all developmental stages, though the levels vary (data not shown). The polyadenylated transcript migrates as a single species of 4.2 kb, relative to DNA size markers. This is consistent with the 4005 bp of the full-length cDNA measured by sequencing.

smo Encodes a Putative G Protein–Coupled Receptor

Conceptual translation of the *smo* cDNA reveals a single large open reading frame encoding a protein of 1036 amino acids with four methionines among its first 14 residues (Figure 6). While the sequence preceding the second ATG of the open reading frame fits best to the Kozak consensus for vertebrate intiation (Kozak, 1987), the sequence preceding the third ATG is a better match to the consensus for initiation of translation in Drosophila (Cavener, 1987). However, none of the four potential translation initiation sites shows a high homology to either consensus sequence. Using the second or third ATG as initiator codon, the predicted protein products consist of 1028 or 1024 amino acids with molecular masses of 115.5 or 115.0 kDa. Hydropathy analysis predicts that the putative Smo protein is an integral membrane protein with seven membrane spanning α helices and a long hydrophilic C-terminal tail (Figure 6). An additional hydrophobic segment near the N-terminus has characteristics of a signal peptide (von Heijne, 1986). Its cleavage would generate an N-terminal extracellular domain that includes five potential N-linked glycosylation sites (Figure 6). Two additional N-linked glycosylation sites are found in the putative first and second extracellular loops between the transmembrane α helices. These structural predictions suggest that Smo belongs to the serpentine receptor family, whose members are all coupled to G proteins (reviewed by Kobilka, 1992; Strader et al., 1994).

While N-terminal extracellular domains of the size found in Smo (226 amino acids after cleavage of the putative signal peptide) are common among G protein–coupled receptors whose ligands are peptides or glycoproteins rather than biogenic amines, the large C-terminal domain of Smo (481 amino acids) is unusual. Nevertheless, it includes five potential phosphorylation sites for PKA that, together with the PKA site of the second intracellular loop, might serve desensitization of

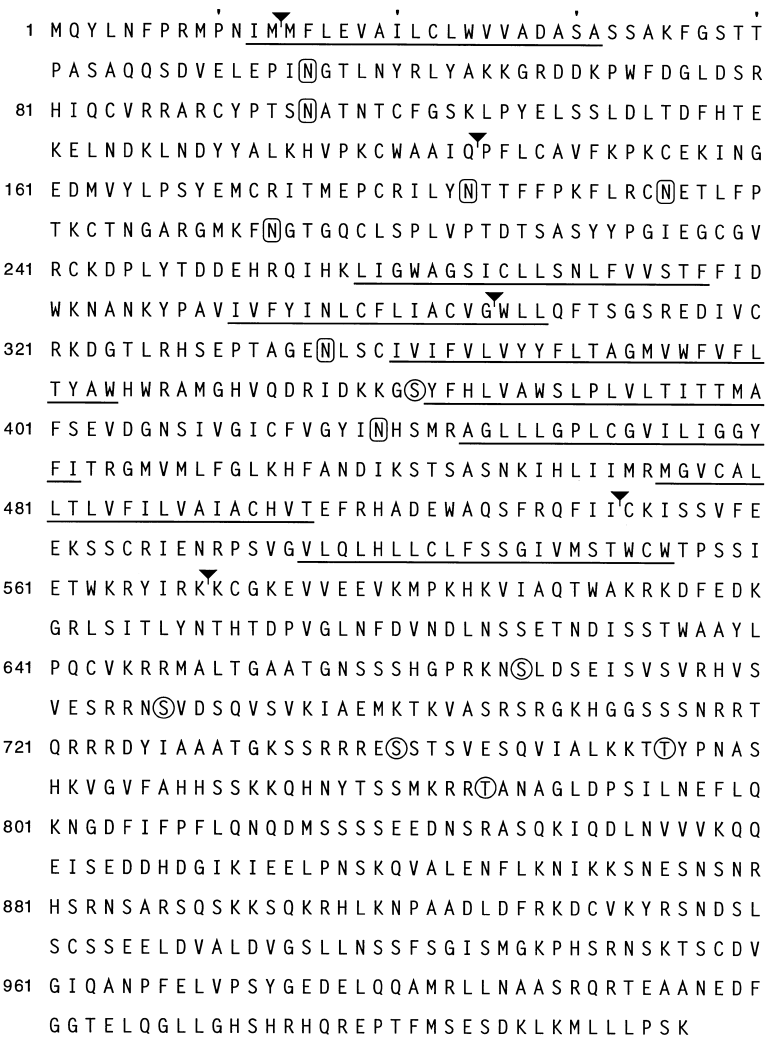


Figure 6. Deduced Amino Acid Sequence of the Smo Protein

The putative amino acid sequence of the Smo protein, derived from cDNA, 5′ RACE, and genomic DNA sequences, is shown. The predicted signal peptide sequence (von Heijne, 1986) and seven transmembrane domains (TMpred program, based on the statistical analysis of the TMbase database [Hofmann and Stoffel, 1993]) are underlined. Seven potential N-linked glycosylation sites are boxed (Gavel and von Heijne, 1990), while six putative PKA phosphorylation sites are circled (Glass et al., 1986). The positions at which the coding region is interrupted by five introns are indicated by triangles. Amino acids are numbered in the left margin.

Smo by its uncoupling from the G_α protein subunit as has been observed for serpentine receptors (Kobilka, 1992).

According to database searches, Smo is most closely related to the Frizzled (Fz) family of transmembrane glycoproteins (Vinson et al., 1989; Chan et al., 1992; Park et al., 1994; Zhao et al., 1995; Wang et al., 1996). Alignment of the Drosophila Smo and Fz sequences in Figure 7 shows extensive homology between Fz and the N-terminal moiety of Smo (29% identity and 46% similarity including conservative substitutions), while the large C-terminal domain of Smo has no counterpart in Fz. The Fz protein thus may be considered a truncated Smo homolog. The homologous domains comprising the seven transmembrane segments and their flanking sequences (235–592 of Smo and 226–581 of Fz) are much better conserved (31% identity and 52% similarity) than the N-terminal hydrophilic domains (25% identity and 37% similarity), shown to be extracellular in Fz (Park et al., 1994). The N-terminal domains include 13 cysteines, of which at least 10 appear to be conserved, and each of the three extracellular loops has one conserved cysteine. As extracellular cysteines are known to play a crucial role in maintaining protein structure by the formation of intramolecular disulfide bridges, this might hint at structural similarities between the extracellular portions of Smo and Fz (Strader et al., 1994).

Discussion

The *smo* gene is required for the maintenance of segmentation in Drosophila embryos (Nüsslein-Volhard et al., 1984). This maintenance depends crucially on segmentally repeated Hh and Wg signaling at the parasegment boundaries. The secreted Hh and Wg protein signals maintain each other's synthesis in neighboring stripes and thereby establish signaling centers, which organize the segmental pattern of the epidermis (Bejsovec and Wieschaus, 1993; Heemskerk and DiNardo, 1994; reviewed by Perrimon, 1994). The lack of segmentation in *smo* mutant embryos might therefore be explained if either Wg or Hh signaling or both depended on smo^+ activity. We have shown here that in *wg* mutant embryos, En decays during stage 9, while in *hh* and *smo* mutant embryos the decay of En protein is delayed until stage 10, clearly after the decay of the Wg signal (Figure 1). These results suggest that transduction of the paracrine Wg signal, which maintains *en* (Siegfried et al., 1992, 1994) and *hh* expression (Eaton and Kornberg, 1990; Domínguez et al., 1996), is not dependent on *smo*.

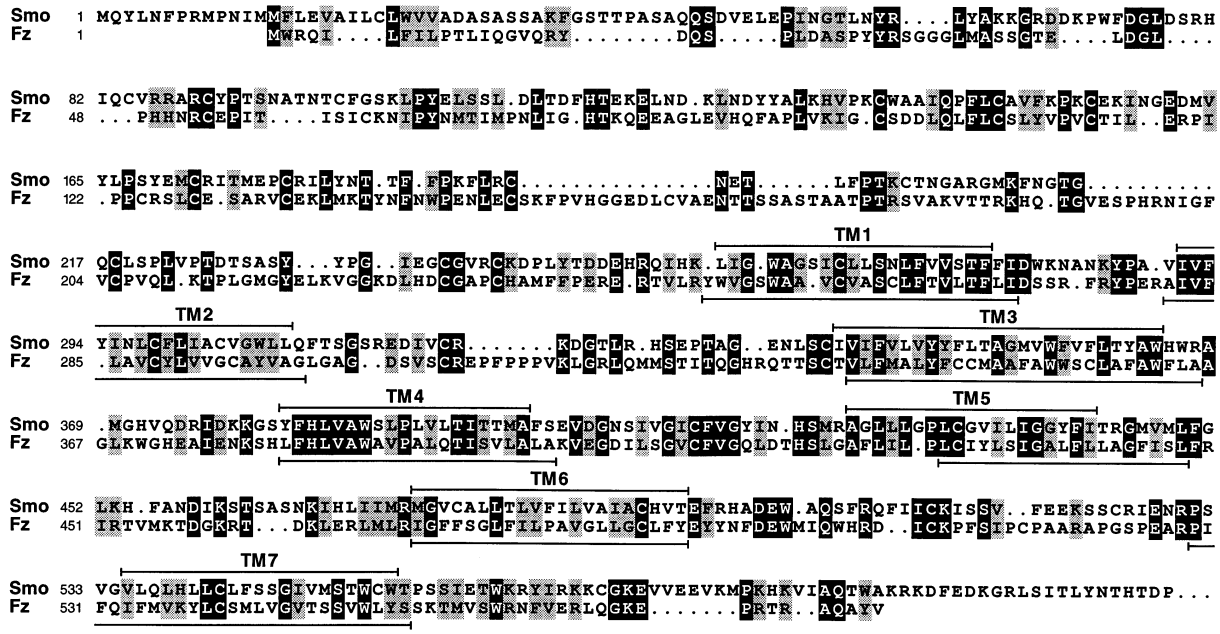


Figure 7. Homology Between Smo and Fz Proteins

The predicted amino acid sequences of the N-terminal portion of the Smo protein and the entire Fz protein from Drosophila melanogaster (Vinson et al., 1989) were compared using the Wisconsin Package, Version 8.0 (Genetics Computer Group, 1994) and the BOXSHADE 3.0 program (Hofmann and Baron, Bioinformatics Group, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Identity of amino acids are shaded in black, similarity in gray. The seven conserved transmembrane domains, predicted by using the TMpred program on either sequence (Hofmann and Stoffel, 1993), are indicated as TM1–TM7.

Instead, *smo* is required for maintenance of *wg* expression during stages 9 and 10, a process that depends on both the Hh signal and an autocrine Wg signal (Hooper, 1994; Yoffe et al., 1995). A pair of additional observations rule out a role of *smo* in paracrine Wg signaling but are consistent with a role in Hh or autocrine Wg signaling. In *smo* mutant embryos, exogenous Wg maintains En expression beyond stage 10 (Figure 2), and exogenous Hh expression is unable to rescue Wg expression (Figure 3).

smo May Encode the Receptor for the Hh Signal

The amino acid sequence encoded by *smo* establishes that Smo belongs to the Fz family of seven-pass–membrane receptors (Figures 6 and 7). Smo could be the Hh receptor or the autocrine, but not paracrine, Wg receptor. If Smo is the autocrine Wg receptor, then an additional Wg receptor would have to transmit the paracrine signal. However, in all studied cases of autocrine and paracrine signaling, signal molecules use the same receptors in both modes. Moreover, in known cases where the paracrine and autocrine pathways differ, they always diverge downstream of their common receptors.

Our recent unpublished results are also inconsistent with the proposal that Smo is an autocrine Wg receptor. Specifically, ectopic expression of Wg activates *gsb* in an autocrine fashion (Li and Noll, 1993) even in the absence of *smo* (data not shown). This activation of *gsb* by Wg does not depend on *smo* and hence excludes *smo* from being in the autocrine Wg signaling pathway. We conclude that Smo is involved neither in paracrine nor autocrine Wg signaling but acts downstream of the Hh signal. Moreover, the finding that *smo* encodes a G protein–coupled receptor is in excellent agreement with the observed role of PKA in Hh signal transduction (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Taken together, all of these observations and considerations argue that Smo is the receptor for the Hh signal.

Ptc was previously proposed to be a constitutively active receptor that was inactivated by binding of Hh, thus permitting transcription of Hh-target genes (Ingham et al., 1991). Specifically, *ptc* is a negative regulator of expression of the Hh-target genes *wg*, *ptc,* and *dpp* and is epistatic to *hh* with respect to these markers (Martinez Arias et al., 1988; Hidalgo and Ingham, 1990; Ingham et al., 1991; Hidalgo, 1991; Sampedro and Guerrero, 1991; Ingham, 1993; Basler and Struhl, 1994; Capdevila et al., 1994; Schuske et al., 1994; Tabata and Kornberg, 1994). However, in the absence of Ptc, the Hh signal still enhances *wg* transcription (Bejsovec and Wieschaus, 1993). It follows that *ptc* is not strictly epistatic to *hh* and thus eliminates the necessity to postulate that Ptc acts as a receptor for the Hh signal. We propose a different role for Ptc: it serves as an accessory protein that might be associated with Smo and regulates its sensitivity to the Hh signal (Figure 8).

A Model for the Transduction of the Hh Signal

The transduction of the Hh signal in the epidermis of embryos and in imaginal discs is very similar (reviewed by Ingham, 1995). Ptc antagonizes the activation by the Hh signal of the target genes *wg* and *ptc* (Ingham et al.,

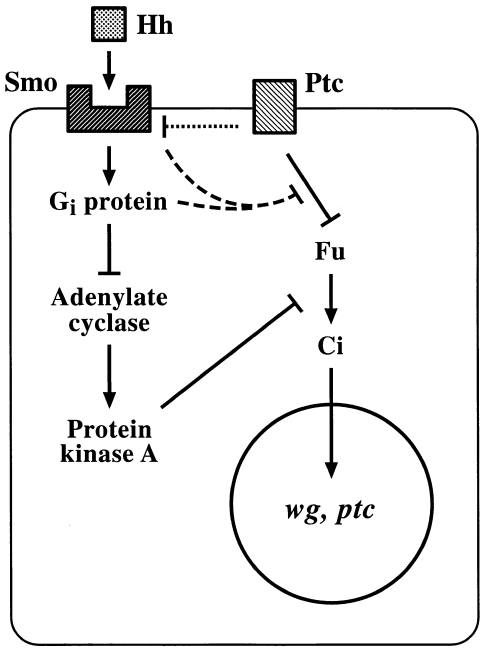


Figure 8. Role of Smo and Ptc in Hh Signal Transduction

Based on the predicted structural characteristics and its position in the Hh signaling pathway, Smo is proposed to transduce the Hh signal through the activation of an inhibitory G_i protein, generating two active signaling moieties, G_α and $G_{\beta\gamma}$ subunits. One of these inhibits adenylate cyclase, leading to a reduction of PKA function, while the other might relieve the Ptc-dependent inhibition of Fu. The net effect is two modes of activation of the zinc-finger protein Ci, which is required in the expression of the Hh-target genes *wg* and *ptc*. Since *wg* is expressed in *ptc hh* mutants while it decays in *smo ptc* mutants, we propose that Smo has a low level of constitutive activity that is negatively regulated by Ptc. This negative regulation of Smo by Ptc might occur by a direct interaction of Ptc with Smo, indirectly by the inhibition of Fu by Ptc, or by a combination of both of these modes. Binding of the Hh ligand to Smo counteracts the inhibition by Ptc. The dotted line indicates a possible interaction between Ptc and Smo, while the dashed lines represent alternative mechanisms by which Smo overcomes the inhibition by Ptc. For a detailed explanation, see text.

1991; Ma et al., 1993; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Schuske et al., 1994; Tabata and Kornberg, 1994). This antagonism between Hh and Ptc is also evident from the very similar phenotypes generated by ectopic expression of *hh* or by loss of *ptc* activity (Phillips et al., 1990; Basler and Struhl, 1994). Activation of the Hh-target genes depends on the activities of Smo (Figure 4), the serine/threonine kinase Fu, and the transcription factor Ci (Hidalgo and Ingham, 1990; Limbourg-Bouchon et al., 1991; Forbes et al., 1993). All three are epistatic to Ptc (Figure 4; Forbes et al., 1993; Hooper, 1994; Motzny and Holmgren, 1995). The concentration of Ci is enhanced by Hh and reduced by Ptc at a post-transcriptional level (Johnson et al., 1995; Motzny and Holmgren, 1995). Similarly, the concentration of Ci is altered in the absence of Fu activity, which shows that Ci acts downstream of Fu in Hh signal transduction (Motzny and Holmgren, 1995). Finally, the *ciD* gain-of-function phenotype in the embryo is epistatic to loss of function for both *hh* and *smo* (T. V. O. and J. E. H., unpublished data).

Another factor known to regulate Hh-target genes is PKA (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Similar to Ptc, PKA inhibits Ci activity as evident from the fact that inhibition or loss of PKA function activates *wg* and *ptc* in the absence of an Hh signal (Jiang and Struhl, 1995; Li et al., 1995; Lepage et al., 1995). Thus, PKA is epistatic to Hh and hence acts downstream or in parallel to Hh signaling (Jiang and Struhl, 1995; Li et al., 1995). Moreover, as Ptc is unable to inhibit transcription of its own gene in *pka*$^-$ clones, PKA is also epistatic to Ptc and thus acts downstream or in parallel to Ptc (Jiang and Struhl, 1995; Li et al., 1995). Neither Hh nor Ptc acts exclusively through PKA, as attested by the following observations. A constitutive level of PKA activity, one that is sufficient to suppress ectopic activation of *ptc* in *pka*$^-$ clones, is unable to counteract the effect of the Hh signal at the anteroposterior border (Li et al., 1995) and does not prevent the ectopic expression of Hh-target genes in *ptc*$^-$ clones (Jiang and Struhl, 1995; Li et al., 1995). That PKA is not the only effector of the Hh signal is also consistent with the observation that loss of PKA activity does not produce all consequences of Hh signaling (Jiang and Struhl, 1995).

These findings, combined with the results reported here, are explained best by the model illustrated in Figure 8. We propose that *smo* encodes a G protein–coupled receptor that responds to the extracellular Hh signal via a trimeric G_i protein inhibiting adenylate cyclase (Figure 8). Ptc and Hh act in parallel on Fu and Ci to regulate transcription of *wg* and *ptc*. Ptc and PKA inhibit activation of *wg* and *ptc* by Ci, while Hh counteracts this inhibition. This is consistent with the observation that Ptc does not act exclusively through PKA (Jiang and Struhl, 1995; Li et al., 1995) but also acts in a parallel pathway.

An important difference between the inhibitory activities of Ptc and PKA is that loss of PKA function activates Hh-target genes in the absence of a functional Hh signal regardless of Ptc concentrations, whereas loss of Ptc function is unable to activate Hh-target genes at high levels of constitutive PKA activity (Li et al., 1995). These findings imply, first, that the ability of Ptc to inhibit activation of Hh-target genes depends on PKA and second, that PKA does not act on Ptc but on a component downstream of Ptc (Figure 8). Recent results show that high PKA activity cannot counteract the phosphorylation of Fu that depends on the Hh signal but can be inhibited by Ptc, suggesting that Fu indeed acts downstream of Hh and Ptc (Thérond et al., 1996). It further follows that PKA does not act on a component between Ptc and Fu. It is unclear, however, whether PKA acts through Fu or a component downstream of Fu. It has been assumed that Hh signaling depends entirely on Fu because maintenance of *wg* expression depends on Fu activity already during stage 9 (Forbes et al., 1993) and because *fu* is epistatic to ectopic Hs-*hh* expression (Ingham, 1993). However, since the early maintenance of *wg* expression depends not only on Hh but also on an autocrine Wg signal (Hooper, 1994; Yoffe et al., 1995), Fu may be required in the autocrine Wg signaling pathway. In this case, the PKA–dependent transduction of the Hh signal

does not have to act through Fu but could converge with the Ptc-dependent pathway at a component below Fu, for example on Costal-2 (Préat et al., 1993) or Ci. Regardless of whether or not PKA acts through Fu, the activation of *ptc* by Ci is determined by the relative activities of PKA and Ptc.

The antagonistic effects exerted by Hh and Ptc are further evident from the observation that both loss of *ptc* function and ectopic Hh expression result in ectopic *wg* activation (Ingham et al., 1991; Ingham, 1993; Tabata and Kornberg, 1994). Moreover, in the absence of both antagonists Hh and Ptc, the Hh signaling pathway is constitutively active because *wg* remains expressed in *ptc hh* double mutants (Ingham et al., 1991). We now find that this activity completely depends on Smo, even in the presence of Hh, since *wg* expression is no longer maintained in *smo ptc* double mutants (Figure 4). It follows that Smo is constitutively active in *ptc hh* embryos. This constitutive activity of Smo is stimulated by the Hh signal (Bejsovec and Wieschaus, 1993) and inhibited by Ptc in the absence of Hh. Because the effects of Ptc and Hh are parallel and linked, as argued above, Hh overrides the inhibition by Ptc by counteracting the inhibitory effects of both PKA and Ptc on Fu and Ci (Figure 8). Ptc might inhibit constitutive Smo signaling by acting through Fu (Thérond et al., 1996). On the other hand, Hh overcomes this inhibition as well as that of PKA.

The simplest possible mechanism by which Hh relieves the inhibition by Ptc takes into consideration the fact that both Smo and Ptc (Hooper and Scott, 1989; Nakano et al., 1989) are integral membrane proteins and assumes that Ptc is inactivated by its association with the Hh–Smo complex. It is also possible that Ptc in its active form is already associated with Smo in the absence of bound Hh (Figure 8). In that case, when Hh binds to the Smo–Ptc complex, Ptc is released from its association with Smo, thus abolishing its inhibitory activity on Fu (Thérond et al., 1996). As a further consequence, PKA is inhibited by G protein–coupled signaling (Figure 8). Alternatively, upon binding to the Smo receptor, Hh might counteract the inhibition of Ptc by generating two active signaling moieties, the G_{α} and the $G_{\beta\gamma}$ subunits that generally have higher and lower affinities, respectively, for their effectors. Either signaling moiety may inhibit the action of Ptc on Fu. Different affinities of G_{α} and $G_{\beta\gamma}$ for their respective effectors would provide a simple explanation for qualitatively different responses to low and high levels of Hh signaling (Heemskerk and DiNardo, 1994).

General Features and Implications of the Model

Several features of our model are new and noteworthy. Smo has constitutive signaling activity (Figure 4) that has been observed only for mutated G protein–coupled receptors (reviewed by Strader et al., 1994). This constitutive activity is inhibited and the sensitivity of Smo for the Hh signal is reduced by the Ptc protein, which plays the role of an accessory protein that modulates the activity of the Smo receptor. Ptc thus restricts the range of the Hh signal by enhancing its threshold concentration for signal transduction through Smo. An analogous situation might exist for the transduction of the Wg signal, in which Nkd appears to restrict the range of the Wg signal by increasing its threshold concentration (Siegfried et al., 1992). Both properties of Smo that are unusual for a receptor coupled to a trimeric G protein, namely its constitutive activity and its inhibition by the accessory protein Ptc, might require additional cytoplasmic domains, which would explain the unusually large cytoplasmic C-terminal moiety of Smo. The balance between the antagonistic Hh signal and Ptc affects at least two parallel pathways (Figure 8). Hh mediates its effect by inhibiting PKA as well as by counteracting the Ptc-dependent inhibition of Fu and possibly of Smo. Thus, Hh acts by antagonizing both PKA and Ptc. Like Hh, Ptc may also play two roles (Figure 8). It inhibits the constitutive signaling of the Smo receptor by direct association and prevents signaling through Fu and Ci.

What would be the advantage of such a complex signaling network? A possible clue might be provided by considering that Ptc on the one hand represses its own activation and on the other hand reduces the sensitivity of the Smo receptor for the Hh signal. As a consequence, Ptc will decay in cells that are not exposed to Hh levels sufficient for the activation of *ptc*. Once the concentration of Ptc is sufficiently low, Hh or the constitutive activity of Smo or both will activate *wg* and *ptc*. As the Ptc concentration rises, the attainable steady state of the Ptc concentration will remain low because of the negative transcriptional feedback exerted by Ptc in the absence of or at very low Hh levels (Figure 8). By contrast, the newly established *wg* transcription is further enhanced and maintained by the *wg–gsb* autoregulatory loop that now, by relying on Wg signaling, is independent of Hh and Ptc (Li and Noll, 1993). Such a mechanism would ensure the delayed anterior expansion of *wg* expression, which mediates the specification of the ventral segmental pattern (Bejsovec and Wieschaus, 1993). The complexity of the signaling network thus would serve to regulate the activation of Hh-target genes with time either by modulating the sensitivity of the Smo receptor through Ptc or by regulating the constitutive activity of Smo. The latter thus might be necessary to prime the *wg–gsb* autoregulatory loop and the anterior expansion of *wg* expression.

These considerations also explain the apparent paradox that Hh activates *ptc* and thus the synthesis of its antagonist. The mechanism of Hh activating *ptc* and Ptc repressing its own synthesis generates different steady-state levels of Ptc, depending on the concentration of Hh. Since Ptc inhibits Smo signaling, the resulting constitutive Smo signaling diminishes as the steady-state concentrations of Ptc increase. The advantage of such a mechanism thus would be to transform a hypothetical Hh signal gradient into a different one of constitutive Smo signaling that might be more stable. It would not be surprising if similar mechanisms are encountered in other systems and organisms.

Experimental Procedures

Drosophila Stocks and Genetics

Three EMS–induced alleles, *smo*^{1}, *smo*^{2}, and *smo*^{3}, were obtained from the Tübingen stock center (Nüsslein-Volhard et al., 1984). Both

the deficiency *Df(2L)dpp^{S4L} Tg^{R}* [21B; 21C6], derived as recombinant from *In(2L)dpp^{S4}* [21B; 22F1–2] and *In(2L)Tg* [21C6; 22F1–2], and the terminal duplication *Dp(2;f)ush^{vx21}* [21A; 21C6], which covers a haplolethality of the deficiency, were generated and kindly provided as *Df(2L)dpp^{S4L} Tg^{R} bw sp/CyO*; *Dp(2;f)ush^{vx21}* stock by Pascal Heitzler (IGBMC, Strasbourg, France). *smo/Df* refers to results obtained with both *Df(2L)dpp^{S4L} Tg^{R}* and *Df(2L)L124*. The *w*; *hGAL4[w+] UASwg^{ls}[w+]/TM3*, *Sb* stock (Wilder and Perrimon, 1995) was received from Elizabeth Wilder (Philadelphia, PA), while *w*; *Hs-hh[w+]/TM3*, *Ser* flies (Ingham, 1993) were obtained from Phil Ingham (London, UK). The *ptc^{H84}* allele, an enhancer trap line with *P[lacZ]* inserted into the 3′ exon of *ptc,* was provided by Corey Goodman (Berkeley, CA). All other stocks were obtained from the Bloomington Stock Center and are described by Lindsley and Zimm (1992).

All Drosophila culture and genetics utilized standard techniques except that all embryos were collected and aged at 18°C to maximize expression of the *smo*$^{-}$ phenotype, with the exception of the *wg^{IL114}* embryos that were collected at 25°C. Embryos were staged by morphology according to Campos-Ortega and Hartenstein (1985). Chromosomes scored as *smo*$^{-}$ in mapping crosses were tested against at least two different *smo* alleles for viability and for the *smo*$^{-}$ cuticle phenotype.

The effect of ectopic Wg expression in *smo* mutant embryos was assayed by crossing *smo^{2} cn bw sp/CyO* virgins with *smo^{3} b pr/+*; *hGAL4 UASwgts/+* males. Progeny were collected for 12–16 h at 18°C, fixed, and assayed for En protein.

The effect of ectopic Hh expression in *smo* embryos was assayed by crossing *smo^{2} cn bw sp/CyO* virgins with *smo^{3} b pr/+*; *Hs-hh/+* males. Progeny were collected at 18°C for 4 hr, aged an additional 6 hr, heat-shocked at 37°C for two 30 min periods separated by 90 min at 18°C, then allowed to recover for 15 min at 18°C, fixed, and assayed for Wg protein.

The *smo ptc* phenotype was determined by collecting embryos from *smo^{1} b pr Df(2R)44CE bw sp/CyO* and from *smo^{1} ptc^{H84} P[w+-lacW] bw sp/CyO* parents at 18°C. The embryos were fixed and assayed for *wg* RNA, Gsb protein, and En protein. Similar results were obtained for crosses among each genotype, where approximately 75% of the offspring showed wild-type expression patterns and the remaining embryos displayed the distinct mutant pattern.

Genomic rescue experiments used the P-element vector pCaSpeR4 (Pirrotta, 1988), *Df(1)w67c2*, *y* host flies, and standard techniques (Rubin and Spradling, 1982). Rescuing activity was defined by restoration of full viability, fertility, and normal morphology to *smo^{1}/ smo^{2}* flies.

Immunohistochemistry and In Situ Hybridization

Detection of Wg protein was performed by staining embryos with rabbit anti-Wg antiserum (van den Heuvel et al., 1993; gift of Roel Nusse, Stanford University). En immunohistochemistry used the 4D9 monoclonal antibody (gift of Tom Kornberg), while Gsb immunohistochemistry used a 1:1 mix of two monoclonals, 10E10 and 16F12 (Zhang et al., 1994; gift of Robert Holmgren, Northwestern University). The primary antibodies were detected with the Vectastain ABC detection system (Vector Laboratories, Burlingame, CA) or with an alkaline phosphatase conjugate of affinity-purified goat anti-mouse IgG H+L (Jackson ImmunoResearch Laboratories, West Grove, PA) according to standard procedures (Kania et al., 1990). Double-labeling of Wg and En proteins was carried out according to Lawrence et al. (1987), with a slight modification. To visualize En as the second label, aminoethylcarbazole (Sigma Chemical Co., St. Louis, MO) was used as a substrate for horseradish peroxidase, which yields an orange product in the presence of hydrogen peroxide (Harlow and Lane, 1988).

In situ hybridizations utilized antisense-strand riboprobes and were performed according to standard procedures (Tautz and Pfeifle, 1989; Jiang et al., 1991).

Isolation and Analyses of Genomic and cDNA

Standard procedures, such as the isolation of genomic DNA, the construction and screening of genomic libraries in EMBL4 vectors, chromosomal walking, whole genome Southern analysis, in situ hybridization to salivary gland chromosomes, and isolation of total and poly(A)$^{+}$ RNA were carried out essentially as described (Maniatis et al., 1982; Frei et al., 1985; Kilchherr et al., 1986).

A cDNA library from poly(A)$^{+}$ RNA from 4–8 hr old *OregonR* embryos was constructed using the Stratagene ZAP-cDNA synthesis kit (Stratagene). cDNAs were subcloned between the *Eco*RI (5′ end) and *Xho*I (3′ end) sites of the plasmid vector pBluescript SK(-). Isolation of cDNA clones was carried out according to standard procedures and following instructions from Stratagene. From a total of 7.5 $\times$ 10^{5} phage clones, 15 independent *smo*-cDNA clones were isolated. To clone a cDNA that includes the transcriptional start site of the *smo* mRNA, the 5′ RACE technique was applied to poly(A)$^{+}$ RNA from 4–8 hr old embryos, using the Amplifinder RACE kit and following instructions from Clontech. A pair of independent RACE products had the same 5′ end, while a third one started two nucleotides downstream of this 5′ end.

DNA sequences were determined on both strands of the *smo* cDNAs and the corresponding genomic DNA with a DNA sequencer model 373A using dye terminators (Applied Biosystems Inc.). DNA sequence analyses and EMBL and protein data bank searches were performed by the use of version 8.0 of the GCG sequence analysis software package (Genetics Computer Group, 1994, Wisconsin) on a Unix system.

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